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Characterization of the TMPRSS2 Protease as a Modulator of Prostate Cancer Metastasis

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14. ABSTRACT
TMPRSS2 is a Type II transmembrane serine protease (TTSP) regulated by androgenic hormones and is expressed preferentially in the human prostate. Although the functional role of this enzyme remains unclear, interest in such a serine protease stems from observations of its clinical implication in prostate cancer, as emerging evidence indicates increased TMPRSS2 expression in prostate carcinoma. To determine the relevance and consequence of TMPRSS2 overexpression in prostate cancer development, we generated four transgenic mouse models that overexpress human or mouse, wild type or mutant forms of TMPRSS2. We have determined that the transgenic lines have significantly increased expression of the target genes, with hrGFP as a transgene marker clearly visible in the ventral lobes while HA expression is detected in all of the prostate lobes. Although enhanced TMPRSS2 expression does not affect mouse fertility, we observed disorganization and disruption of the basement membrane. These findings suggest a mechanism by which TMPRSS2 contributes to primary prostate cancer progression and promotes metastasis.

15. SUBJECT TERMS
TMPRSS2, serine protease, transgenics, carcinoma, pathophysiology, metastasis

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INTRODUCTION:

Members of serine protease gene family carry out diverse roles in normal mammalian physiology, and contribute to the pathogenesis of disease. Recently serine proteases expressed in prostate cells have emerged as important molecules for understanding the pathophysiology and mechanisms of prostate cancer metastatic progression. The enzymatic activities of these proteases when expressed in ectopic locations such as in the bone may alter the local environment to favor tumor cell invasion and growth. Transmembrane serine protease II (TMPRSS2) is a membrane-bound serine protease that is regulated by androgenic hormones and is expressed preferentially in the human prostate. We have recently shown that TMPRSS2 expression is increased in prostate carcinoma and is expressed in the majority of metastatic prostate cancer foci. To date, the potential for TMPRSS2 to contribute directly to the invasive and metastatic events that occur during the process of prostate carcinogenesis has not been reported. I hypothesize that progression to metastatic prostate cancer can be driven by the persistent *in situ* and ectopic expression of TMPRSS2 by prostate cancer cells; that TMPRSS2 influences invasion and metastasis through protease action on barriers to invasion (e.g. extracellular matrix, endothelium) and reciprocal alterations involving signaling factors derived from the tumor and microenvironment (e.g. (Insulin growth factor (IGF), Hepatocyte Growth Factor (HGF), Chemokine CXC motif ligand (CXCL12)).

BODY:

The following summarizes the technical objectives for the proposal and the work accomplished during the 12-month interval between the project initiation (03/01/08) and report preparation (03/01/09).

Training and Career Development: The training and career development components of this research fellowship have included the acquisition of several laboratory bench skills as well as the enhancement of my communication and presentation skills. In the laboratory, I have received training in human and mouse histology/pathology from Dr. Larry True, a pathologist with expertise in prostate cancer. I have learned laser capture microscopy, tissue culture, tissue recombination and xenograft techniques. I have attended the weekly Nelson laboratory meetings and presented my research findings quarterly, receiving feedback from Dr. Nelson and members of the Nelson lab. I have presented my research findings to the faculty and trainees of the Hutchinson Center through the Friday Night Research in Progress Seminar Series. I have attended the weekly Program in Prostate Cancer Research meetings and interacted with visiting faculty on a regular basis. I have also received training in the responsible conduct of research and research ethics through the scheduled seminars and training conducted jointly through the Fred Hutchinson Cancer Center and the University of Washington. Finally, I have met with Dr. Nelson twice per year to review my overall progress and develop career objectives and plans for the future.

Specific aim 1. **Determine the biological roles and molecular mechanisms by which TMPRSS2 influences primary prostate tumor growth and metastasis**. (Months 1st-18th)

*Task 1a (**Completed**): Examine if TMPRSS2 has the potential to influence prostate cancer cell characteristics in an in vitro 3D environment, by focusing on proliferation, adhesion, and invasion. (Months 1-2).*

To date, we found activated TMPRSS2 can cleave HGF, and increases migration and invasion of DU145 prostate cancer cells tested through transwell culture. We observed no changes in proliferation or adhesion.

To model the *in vivo* functions of human TMPRSS2, we initiated biochemical studies to examine its substrate specificity in detail, and to determine the potential roles of TMPRSS2 in prostate cancer cells. First, we focused the search for macromolecular protein substrates on precursor forms of extracellular serine-protease-fold zymogens, whose expression was coincident with TMPRSS2 in prostate tumors and/or normal tissues. Such a precursor has to become activated by characteristic proteolytic cleavage events that take place in their activation domains. In our study, we found that TMPRSS2 cleaved the precursor of hepatocyte growth factor sc-HGF (single-chain HGF) *in vitro* (**Figures. 1A; 1B**); and the cleaved HGF induced auto-phosphorylation of the HGF receptor (c-Met) in DU145 cells (**Figure 1C**), indicating that HGF is processed specifically and is functionally active. Thus, sc-HGF may be a preferred endogenous substrate for TMPRSS2 and provide a better understanding of how up-regulation of TMPRSS2 expression may influence tumorigenesis.

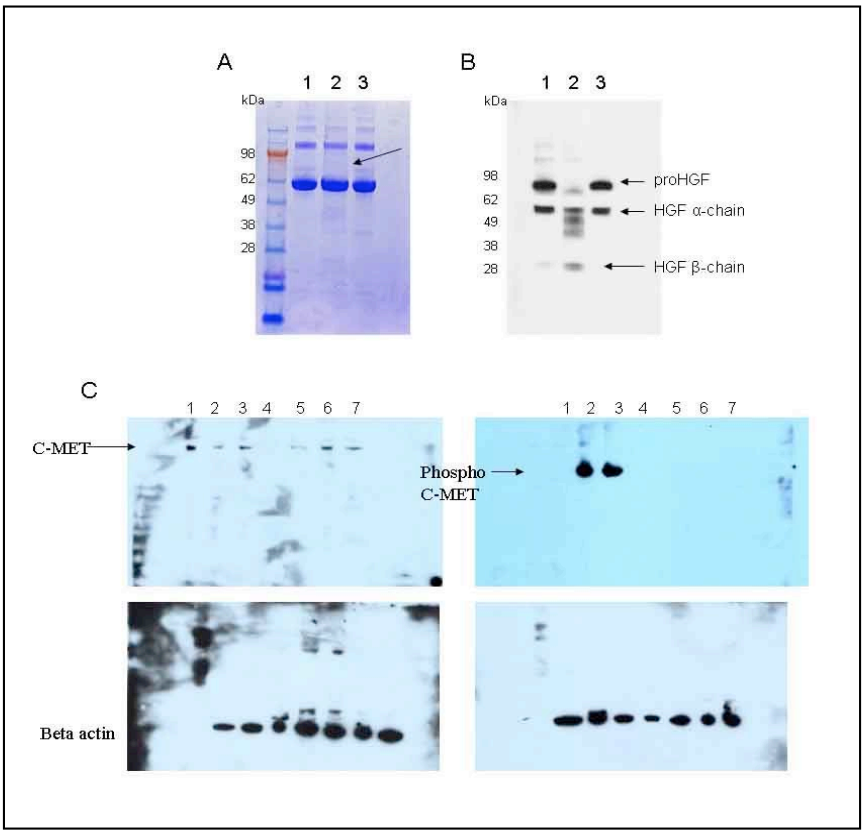

Figure 1. *TMPRSS2 Cleaves Hepatocyte Growth Factor (HGF).* TMPRSS2 was incubated with recombinant HGF (R&D Systems) for 6 hours at 37°C. **A**. Coomassie blue stained gel; **B.** Western blot by antibody against HGF. In either A or B, the loading order was: 1. HGF; 2. HGF with TMPRSS2; 3. HGF with inactive protease domain construct. Note the loss of the proHGF band (arrow) and obvious HGF cleavage products on the Western Blot. **C.** HGF activated by TMPRSS2 causes c-Met phosphorylation in DU145 cells. On each blot: 1. Serum free; 2. HGF + matriptase; 3. HGF + TMPRSS2; 4. HGF + anti-HGF; 5. HGF + TMPRSS2 + anti-HGF; 6. HGF; 7. 10% FBS. The upper left blot was immunoblotted with anti-c-met, upper right probed with anti-phospho-c-met, lower left and lower right tested with anti-βactin.

To determine if TMPRSS2 has any effects on cell proliferation and invasion, we transfected the prostate cell lines BPH1, RWPE, PC-3 and DU145 with a TMPRSS2-encoding construct.

Upon overexpression of TMPRSS2, we measured the cell proliferation, adhesion and *in vitro* invasion capacities. As compared vector control, TMPRSS2 increased the migration and invasion of RWPE and BPH1 cells (**Figure 2A**). TMPRSS2 also increased the growth of PC-3 and DU-145 anchorage independent growth (**Figure 2B**). However, overexpression of TMPRSS2 did not alter the proliferation of DU-145 cells when grown on plastic substrate (**Figure 2C**).

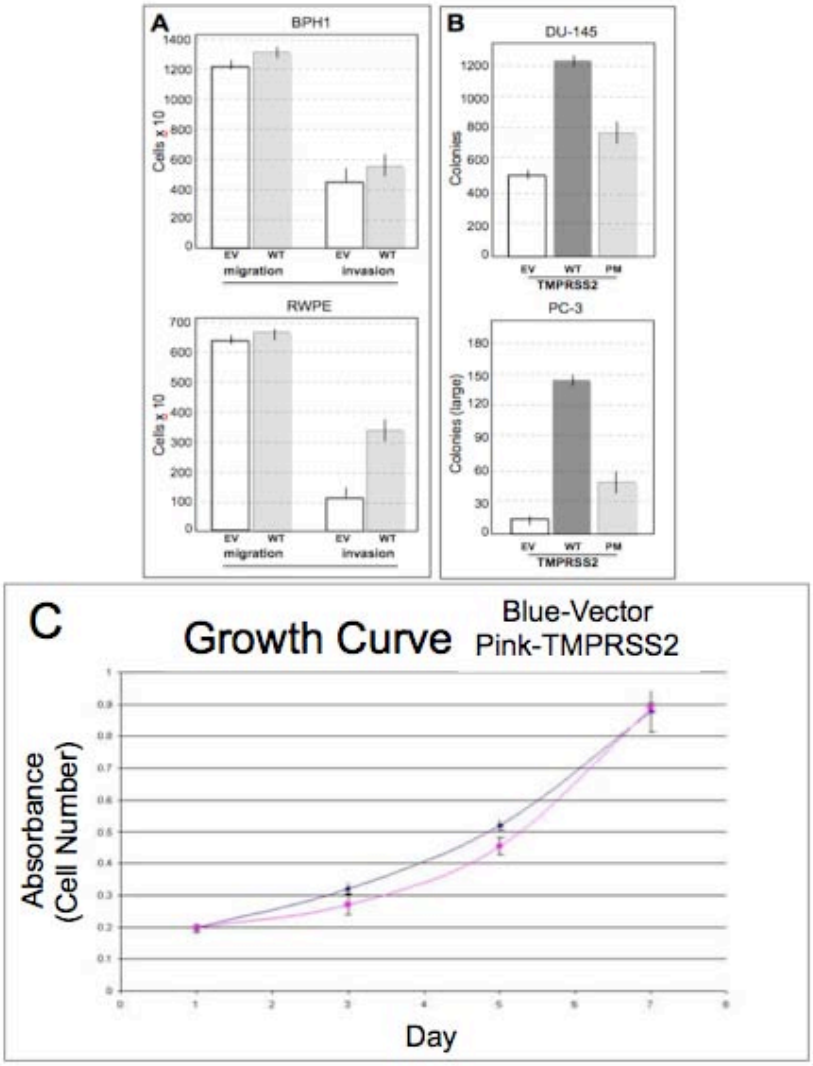

Figure. 2. *Influence of TMPRSS2 expression on cellular phenotypes*. Prostate cell lines were transfected with TMPRSS2 construct or empty vector as controls. **(A)** Expression of TMPRSS2 (WT) increased cellular migration and invasion relative to vector (EV). (B) Expression of wild-type TMPRSS2 (WT) increased anchorage independent growth relative to vector (EV) or mutant inactive TMPRSS2 (PM). **(C)** Expression of TMPRSS2 did not alter the proliferation of DU-145 cells.

*Task 1b (**In progress**): Determine if loss of TMPRSS2 expression influences the development and progression of primary prostate cancer in a characterized model of prostate cancer progression that arises through mechanisms distinct from the Rb/p53 alterations of the TRAMP model.* (Months 3-8). These mouse crosses have been initiated and studies of the TRAMP; TMPRSS2-/- mice is in progress.

*Task 1c (**In progress**): Cross the Tmprss2$^{-/-}$ animals with a mouse model harboring a mutant androgen receptor (AR^{E231G}), and generate litters of Tmprss2$^{-/-}$; AR^{E231G} animals. (Months 8-10).* Due to issues with strain background influences on phenotypes, we are now back-crossing the Tmprss2-/- mice into a homozygous strain background to allow for the accurate interpretation of Tmprss2 influences.

*Task 1d (**In progress**): Evaluate cohorts at various time points for tumor size, differentiation (histology), proliferation and apoptosis.* (Months 11-18)-pending. As above, due to issues with strain background influences on phenotypes, we are now back-crossing the Tmprss2-/- mice into a homozygous strain background to allow for the accurate interpretation of Tmprss2 influences on tumor behavior.

Specific aim 2. **Establish transgenic mice overexpressing human (or mouse) TMPRSS2 (PB-TMPRSS2) and determine if enhanced levels of TMPRSS2 influence cellular differentiation, proliferation, and apoptosis; and if the prostates from these animals display degradation of extracellular matrix and disorganization of the basement membrane as observed with other serine proteases.** (Months 6-20)

*Task 2a (**Completed**): Construct a vector encoding PB-TMPRSS2-IRES-Luciferase sequence, which directs expression of TMPRSS2 transgene to prostate epithelium.* (Months 6-8).

Based on the expression vector (PB-IRES-Luciferase) supplied by the Greenberg lab, our studies indicated that the efficacy of this vector in driving transgene expression was poor, and TMPRSS2 was not expressed to a detectable level. Thus, we adjusted the plan and switched to a 2nd option utilizing an ARR2-PB-hrGFP transgenic plasmid, which was generously provided by Dr. Robert Matusik (Vanderbilt Prostate Cancer Center). This expression vector contains a duplicate synthetic probasin promoter located at 5' upstream of the multiple cloning sites, is androgen-responsive and specifically active in the epithelial cells of prostate, and has been used for prostate-targeted expression of Hepsin (Zhang et al., 2000). The IRES-hrGFP sequence is used to reinitiate the translation of the reporter *renilla* GFP from the same transcript, with the simian virus 40 (SV40) polyadenylation sequence to stabilize the mRNA. Such a configuration drives the enhanced expression of target genes and hrGFP from the same transcript, allowing for timely surveillance of gene expression by whole mount GFP expression.

Upon generation of these transgenic constructs, the functionality of transgenic elements was tested in LNCaP human prostate carcinoma cells by transient transfection with the individual plasmids. Western blot analysis of total protein extract from TMPRSS2-transfected cells using anti-HA antibody revealed a 60 kDa band corresponding to the pro-form of TMPRSS2, while the 30 kDa and 25 kDa represent the cleaved forms of activated enzymes; however, the mutants only exist as a single band at 60 kDa, indicating the self-cleavage abilities of both human and mouse TMPRSS2 are abrogated (**Figure 3**). In parallel, anti-TMPRSS2 pinpointed the presence of both exogenous and endogenous versions (55, 25, 20 kDa, respectively) of these serine proteases, with a molecular weight increase for either pro-form or excised forms of exogenous proteins at approximately 5 kDa as compared with endogenous proteins, consistent with the calculated

weight gain of a fusion peptide including the 3×HA fragment following the C-terminal of TMPRSS2. These data also demonstrated that an attached tail preserved the serine protease activity of TMPRSS2 as an extracellular function.

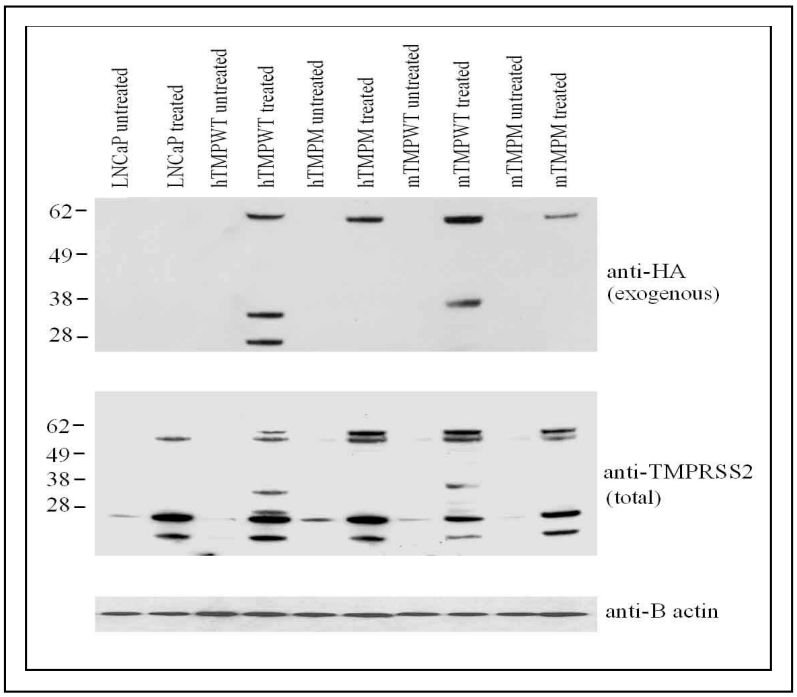

Figure 3. ARR2PB-TMPRSS2 transgenic constructs allow increased expression of TMPRSS2 proteins in a prostate carcinoma cell line in response to androgen stimulation. Each of these constructs was transiently transfected into LNCaP cells, with or without treatment with 10nM R1881, with total protein extracts analyzed by Western blot with anti-HA and anti-TMPRSS2 antibodies sequentially. Anti-HA identifies the component of TMPRSS2 derived from the transgenic constructs as an exogenous source of protein expression. hTMWT is human TMPRSS2 wild type; hTMPM is human TMPRSS2 with a mutated inactive protease domain; mTMWT is mouse Tmpress2 wild type; mTMPM is mouse Tmprss2 with mutated inactive protease domain. Treated designates cell exposed to the synthetic androgen R1881

*Task 2b (**Completed**): Microinject PB-TMPRSS2 fragments into fertilized C57BL/6JxCBA mouse eggs that will be transplanted into pseudopregnant females, and screen the resulting mice by PCR with primers used for amplification of a region encompassing the 3' terminal TMPRSS2 and 5' terminal of the tag sequence.* (Months 9th-12th).

After transgenic delivery to the fertilized eggs and subsequent transplantation into pseudopregnant females, the F1 pups were analyzed by PCR. Four positive human TMPRSS2 (hTW) animals were produced. Although all the pups from the same litter turned out to be positive, some exhibited low TMPRSS2 expression and were not further expanded; in parallel, 3 of 8 human mutant TMPRSS2 (hTM) animals were found to be positive and picked for further analysis. For mouse Tmpress2 (mTW), and mutant mouse Tmprss2 (mTM), we propagated 3 out of 7 and 3 out of 9 positive offspring, respectively. Each of these lines was crossed with WT B6 as breeder pairs that allow for continued line maintenance.

To establish whether the transgenic construct also expresses functional hrGFP protein which serves as an in vivo expression marker, whole mount organ internal GFP detection was performed with fluorescence microscopy with UV light attachment. The hrGFP was readily detectable in the ventral lobe of a prostate gland of the A2-TMP animals (**Figure 4**). Although

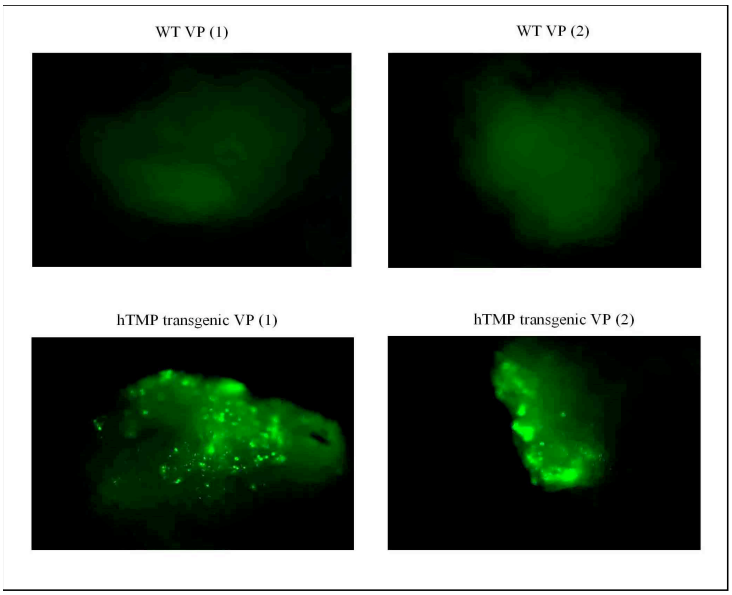

Figure 4. GFP-imaging of prostates from wild-type (WT) and A2-TMPRSS2 animals (hTMP) dissected at age 16-weeks. Note prominent GFP fluorescence in the ventral prostate (VP) lobes of the TMPRSS2 transgenic mice.

hrGFP fluorescence was visible by whole mount organ GFP analysis, it was too weak to be seen on frozen sections. Similarly, other mouse transgenics including hTM/mTW/mTM also exhibited this phenotype.

We collected several different organs and examined the expression of the TMPRSS2 transgene by western blot (**Figure 5**). Within this group of mouse organs, only the prostates of transgenics have detectable signal, as demonstrated by anti-HA staining, and anti-TMPRSS2 antibody (clone P5H9-A3, Lucas et al., 2008) confirmed the difference between wild type and transgenic genotypes.

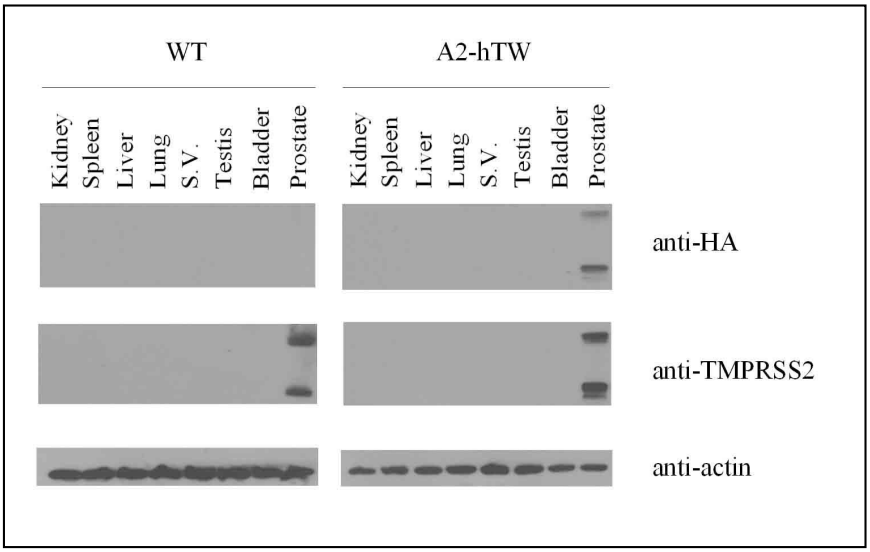

Figure 5. Comparison of TMPRSS2 expression among mouse organs. The A2-hTW transgenic mouse line and wild type line were dissected and 8 difference organs were collected for protein analysis. Total protein was extracted from indicated organs of 16-week-old animals and analyzed by Western blot. β-actin was used as loading control. TMPRSS2 expression was only observed in the prostate gland of the A2-hTW transgenic mice.

Immunohistochemical staining of 4-month old WT and hTW mouse prostate glands with anti-HA antibody revealed histological differences. There was positive staining in the hTW tissues, while WT littermates were essentially negative (**Figure 6**). Although hrGFP was found to be expressed in the ventral lobe, expression of HA as a transgene marker was observed in all the lobes, but at different signal intensities. Among them, VP has a relatively higher staining strength.

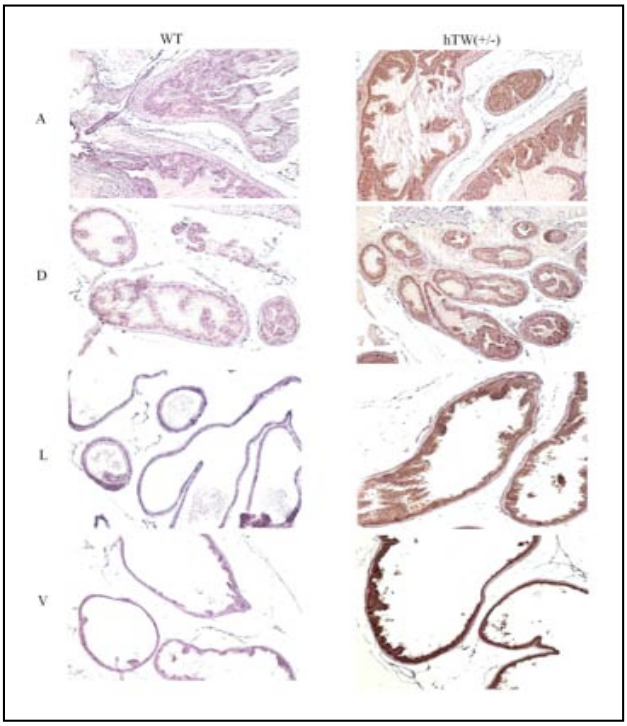

Figure 6. Immunohistochemical analysis of TMPRSS2 expression in wildtype (WT) versus TMPRSS2-transgenic (hTM+/-) prostate glands. A=anterior; D=dorsal; L=lateral; V=ventral. Note pronounced TMPRSS2 immunoreactivity (brown) (based on HA tag expression) in the TMPRSS2 transgenic prostates versus the wild-type control prostate glands.

Given the high and predominant expression of TMP in the prostate, we asked whether the overexpression of TMPRSS2 had an effect on mouse fertility. Breeding F1 generation with WT mice produced litters with genotypes indicative of Mendelian pattern inheritance. In addition, both male and female founder F1 A2-TMP mice produced litter sizes comparable to those of WT controls (**Figure 7**).

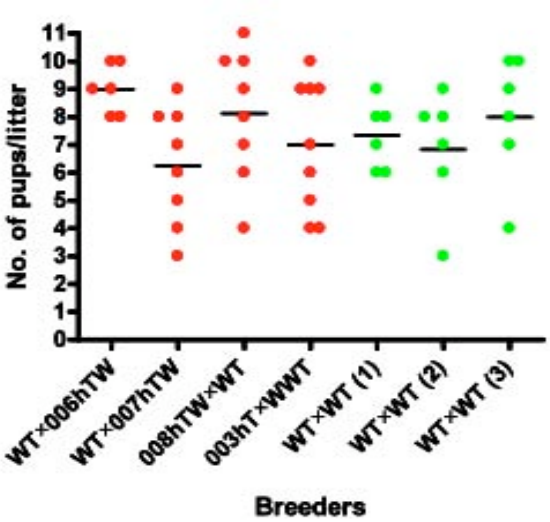

Figure 7. Comparison of litter sizes between wild-type (WT) and Tmprss2 transgenic (TW) animals. No significant differences were observed.

According to earlier reports from the Vasioukhin lab (Klezovitch et al., 2004), PB-hepsin transgenic mice undergo normal differentiation, proliferation, and apoptosis, but display disorganization of the basement membrane. Interestingly, enhanced expression of hTW caused similar pathological consequence, which is histologically apparent starting from the age of 5 months by preliminary examination (**Figure 8**). This result requires further confirmation.

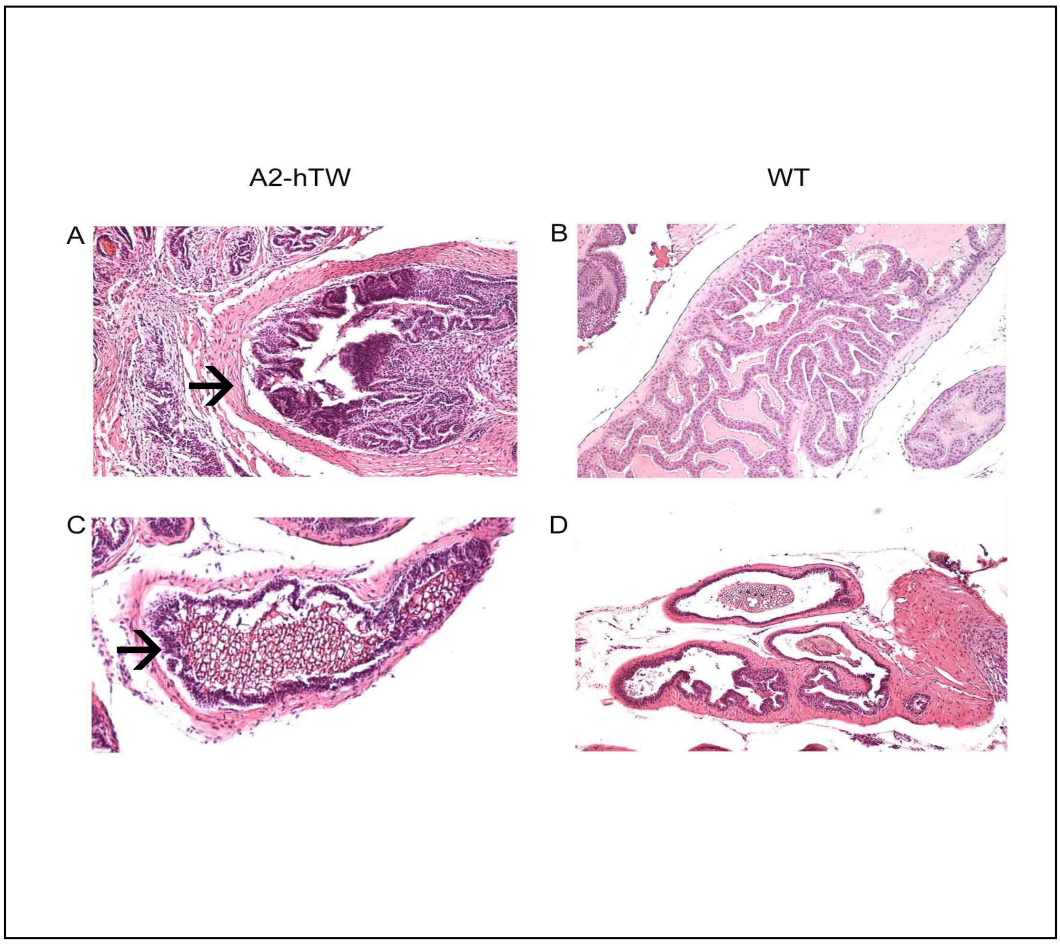

Figure 8. Histologic appearance of prostate lobes from A2-hTW (**A** and **C**) and control (WT) (**B** and **D**) animals. Tissue sections were stained with hematoxylin (H) and eosin (E) and examined using 10× (**B** and **D**) and 20× (**A** and **C**) objectives. Note separation between epithelial cells and the stromal layer in the A2-hTW mouse tissue (arrows, A,C)

*Task 2c (**In Progress**): Cross mice overexpressing TMPRSS2 with mouse strains that develop PIN or invasive but non-metastatic primary tumors, and determine if TMPRSS2 promotes particular events in the metastatic cascade. (Months 12th-20th)*-pending. Crosses with mouse prostate models that develop PIN and invasive cancers have been initiated.

KEY RESEARCH ACCOMPLISHMENTS

- We have demonstrated that activated TMPRSS2 promotes the migration and invasion of prostate cancer cells *in vitro* through enzymatic cleavage of HGF, but not proliferation.
- We constructed a group of expression vectors encoding both wild-type and mutant human TMPRSS2, and wild-type and mutant mouse Tmprss2. These constructs allow directed expression of TMPRSS2 in prostate epithelium.
- We demonstrated that LNCaP cells significantly express the individual transgenes in response to androgen stimulation.

- We microinjected ARR2PB-TMPRSS2 fragments into fertilized C57BL/6JxCBA mouse eggs which were subsequently transplanted into pseudopregnant females, and obtained a group of genetically positive animals.
- We found hrGFP expression was readily detectable in the ventral lobe of a prostate gland of the A2-TMP animals and exogenous TMPRSS2 is specifically overexpressed in mouse prostates (relative to other mouse tissues that were not targeted for expression).
- We demonstrated that enhanced expression of TMPRSS2 had no effect on mouse fertility.
- Preliminary studies identified the degradation and disruption of basement membranes caused by TMPRSS2 overexpression. This is a potential mechanism through which TMPRSS2 promotes cancer metastasis.

REPORTABLE OUTCOMES

None.

CONCLUSIONS

The research accomplished to date has demonstrated that TMPRSS2 has the potential to function as regulator of signaling events at the cell surface interface with the tumor microenvironment. These may include interactions with matrix components, adjacent cells, and activation (or inactivation) of growth factors such as HGF. Both the transgenic marker and target protein level of these mouse lines allow the monitoring of the organ- and age-specific expression of TMPRSS2 *in vivo*. However, overexpression of Tmprss2 in the prostate glands does not influence mouse fertility. Following normal gland development, fragmentation and perturbation of the basement membrane occurred in the prostates of transgenic mice. These results suggest that as with Hepsin expression, TMPRSS2 overexpression may influence prostate cancer progression and enhance metastasis. This possibility will be tested by crossing our ARR2PB-TMPRSS2 mice with $Nkx3.1^{-/-}$ mice that develop PIN, but not invasive cancers, and with LPB-Tag (Line 12T-7f) mouse model of prostate cancer, an established model of invasive, but non-metastatic prostate cancer.

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APPENDICES

None.